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Rediscovery and phylogenetic analysis of the Shelta Cave Crayfish (*Orconectes sheltae* Cooper & Cooper, 1997), a decapod (Decapoda, Cambaridae) endemic to Shelta Cave in northern Alabama, USA

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Abstract

The Shelta Cave Crayfish (*Orconectes sheltae*) is a small, cave-obligate member of the genus *Orconectes* (family Cambaridae) endemic to a single cave system—Shelta Cave—in northwest Huntsville, Madison Co., Alabama, USA. Although never abundant, this stygobiont was regularly observed in the 1960s and early 1970s before the population and aquatic community in general at Shelta Cave collapsed likely in response to groundwater contamination and the loss of energetic inputs from a Grey Bat (*Myotis grisescens*) maternity colony that abandoned the cave after installation of a poorly designed cave gate. We conducted 20 visual surveys of aquatic habitats at Shelta Cave between October 2018 and July 2021. Although the aquatic community has not recovered, we did confirm the continued existence of *O. sheltae*, which had not been observed in 31 years, with observations of an adult female on 31 May 2019 and an adult male on 28 August 2020. We conducted the first phylogenetic analyses of *O. sheltae* and discovered that the species is most closely related to other geographically proximate stygobiotic crayfishes in the genus *Cambarus* in northern Alabama than members of the genus *Orconectes*. We advocate for recognition of this species as *Cambarus sheltae* to more accurately reflect evolutionary relationships of this single-cave endemic and offer recommendations for its management, conservation, and future research, as this species remains at high risk of extinction.

Keywords

Cambarus, conservation, Crustacea, Decapoda, endangered, Malacostraca, phylogeny, population decline, short-range endemism

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Introduction

Shelta Cave (Alabama Cave Survey no. AMD4) located in northwest Huntsville, Madison County, Alabama, USA was once considered one of the most biologically diverse cave systems not only in the United States but globally (Culver and Sket 2000). Twenty-four cave obligate taxa have been documented historically at Shelta Cave, including 12 troglobionts and 12 stygobionts (Cooper 1975; Hobbs and Bagley 1989; Culver and Sket 2000). The decapod fauna was exceptionally diverse with three stygobiotic crayfishes – Southern Cave Crayfish (*Orconectes australis* (Rhoades, 1941)), Alabama Cave Crayfish (*Cambarus jonesi* Hobbs & Barr, 1960), and Shelta Cave Crayfish (*O. sheltae* Cooper & Cooper, 1997) – and one shrimp – Alabama Cave Shrimp (*Palaemonias alabamae* Smalley, 1961). However, the aquatic fauna experienced precipitous declines in the early 1970s (Cooper 1975), which has been attributed to gating of the entrances leading to the extirpation of a Grey Bat (*Myotis grisescens* Howell, 1909) summer maternity colony that provided an important energy input for the aquatic ecosystem and groundwater pollution associated with increased urbanization (Hobbs and Bagley 1989; Wilson and Robison 1993; McGregor et al. 1997; Culver 1999; Elliott 2000, 2012). Several species, including the Tennessee Cave Salamander (*Gyrinophilus palleucus* McCrady, 1954), *P. alabamae*, and *O. sheltae*, have not been observed for several decades and have been presumed extirpated from Shelta Cave (Elliott 2005; Cooper and Cooper 2011; USFWS 2016).

Orconectes sheltae is a small, stygobiotic crayfish endemic to Shelta Cave. This rare crayfish was discovered in August 1963 after examining specimens collected for experimental studies but was not formally described until 1997 (Cooper and Cooper 1997; holotype, allotype, and morphotype are accessioned in the North Carolina State Museum of Natural Sciences with additional paratypes accessioned in the National Museum of Natural History). *Orconectes sheltae* co-occurs with *C. jonesi* and *O. australis*, but can be readily distinguished from these two crayfishes, which were historically much more abundant than *O. sheltae* (Cooper 1975; Cooper and Cooper 1997), by examination of chelae, gonopods (in form I males), and body size. *Orconectes sheltae* has narrow, elongate chelae with a long palm and that lacks conspicuous setae, the gonopod terminates in two elements – one with a twist corneous central projection and the other with a noncorneous mesial process, and body size is smallest of three species ranging 13.5–19.7 mm total carapace length of 19.7 mm (Cooper 1975; Cooper and Cooper 1997; Buhay and Crandall 2009). In *C. jonesi*, the chelae are larger, robust, and covered with conspicuous setae, the gonopod terminates in two strongly recurved elements with a corneous central project and a tapering noncorneous mesial process, and body size is intermediate between *O. sheltae* and *O. australis* ranging 15.0–23.9 mm total carapace length at Shelta Cave (Cooper 1975; Buhay and Crandall 2009). In *O. australis*, the chelae are larger but not as robust as in *C. jonesi* and also not conspicuously covered in setae, the gonopod terminates in two acute elements – a corneous central projection that is flattened basally and a noncorneous mesial process, and body size is largest of three species ranging 21.0–47.2 mm total carapace length at Shelta Cave (Cooper 1975; Buhay and Crandall 2009).

Orconectes sheltae was uncommon even before the decline in the aquatic fauna at Shelta Cave. Only 18 individuals (15 specimens collected and 3 observed) were observed over nine trips conducted between December 1963 and July 1968 (Cooper and Cooper 1997). An additional 97 individuals were observed during a long-term mark-recapture study between November 1968 and July 1975 (Cooper 1975). The most recent confirmed sighting occurred on 8 December 1988 when a form I male was captured and released in the East Hall section of the cave (Hobbs and Bagley 1989). Despite several biosurveys of the aquatic fauna conducted over the past 30+ years, no additional observations are known (Rheams et al. 1992; McGregor et al. 1994; Miller 2013). *Orconectes sheltae* is State Protected in Alabama under 220–2-.98 (Invertebrate Species Regulation) and a Priority 1 Species (Highest Conservation Concern) under the State Wildlife Action Plan (Alabama Department of Conservation and Natural Resources 2015). This species has been assessed as Critically Endangered B2ab(iii) on the IUCN Red List (Schuster et al. 2010) and Critically Imperiled (G1) by NatureServe (NatureServe 2021). However, the lack of any confirmed observations at Shelta Cave or discovery of new populations over the last 30+ years despite additional surveys has led to concerns that the species may be extinct (Buhay and Crandall 2005; Elliott 2005; Niemiller and Taylor 2019).

Here we report on the rediscovery of *O. sheltae* at Shelta Cave for the first time since 1988. We also summarize available data on cave crayfish counts at Shelta Cave over a nearly 60-year period since 1963. We also provide the first phylogenetic analysis of *O. sheltae* and propose placement of the species in the genus *Cambarus* (subgenus *Aviticambarus*) with several other cave-obligate *Cambarus* species that occur in northern Alabama.

Methods

Study area

The two vertical entrances to Shelta Cave are located within a large sinkhole in a residential area in northwest Huntsville, Alabama within the Highland Rim physiographic province and Tennessee River Watershed in northern Alabama. The 762-m cave system trends in an east-west direction under Cave Avenue, Pulaksi Pike, and several residential homes. Shelta Cave was purchased in 1967 by the National Speleological Society (NSS) to protect and preserve the diverse cave community for scientific research and conservation (Hobbs and Bagley 1989; Culver 1999). The cave is still owned and maintained as a preserve by the NSS. Shelta Cave is developed within the Mississippian-aged Tuscumbia Limestone and Fort Payne Chert undifferentiated (Rheams et al. 1992) and is characterized by three large rooms that are interconnected by short passageways (Fig. 1A). For detailed descriptions of Shelta Cave see Johnston (1933), Torode (1973), Cooper (1975), Hobbs and Bagley (1989), and Rheams et al. (1992). The largest room is Jones Hall measuring ~201 m east-west × 250 m north-south that consists of two main levels: an upper level with a substantial accumulation of breakdown slabs on the

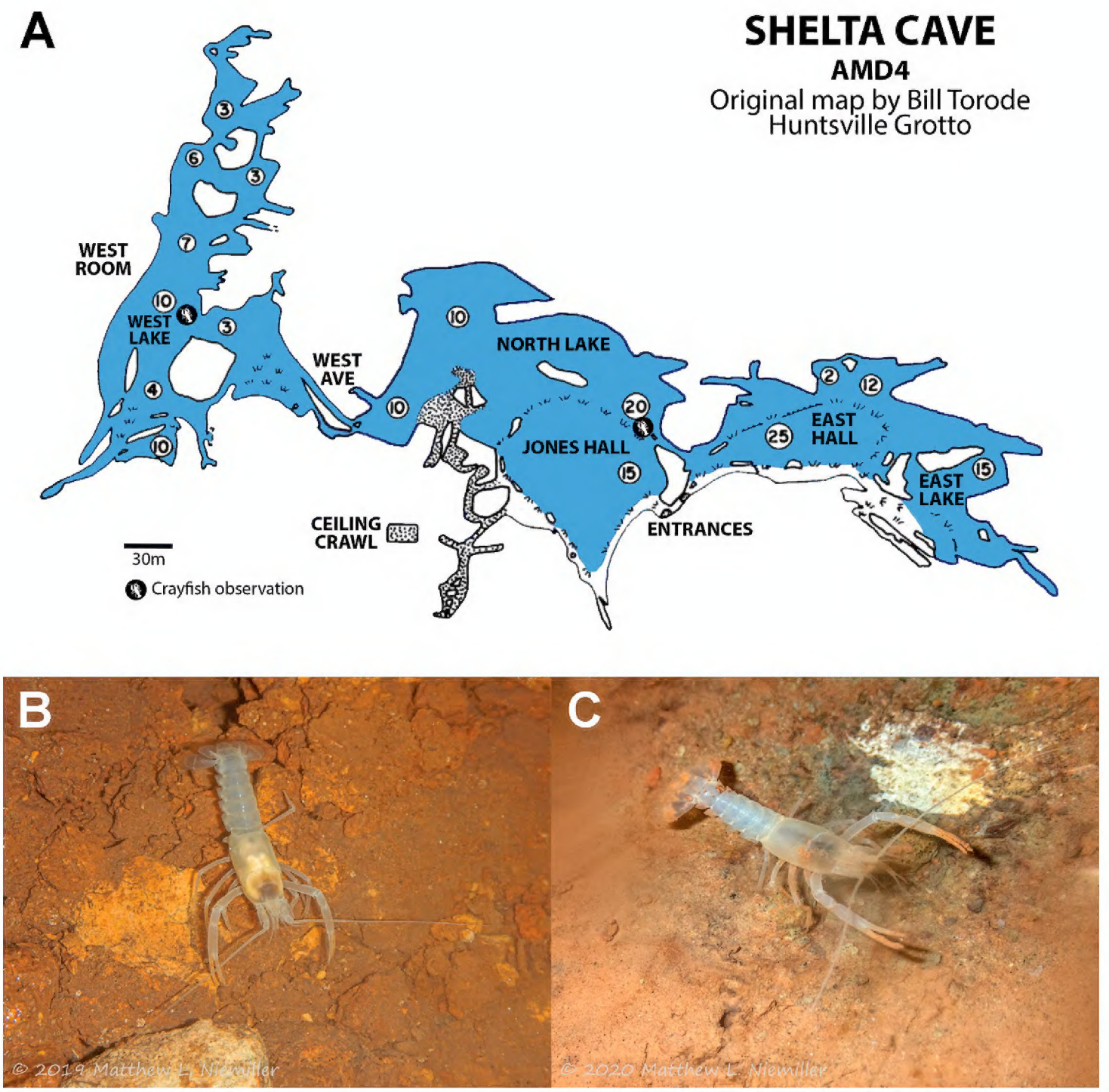

Figure 1. A Map of Shelta Cave showing the distribution of aquatic habitat during high water levels and the location of Shelta Cave Crayfish observations (black crayfish symbol) during the study including **B** a female in 2019 from North Lake and **C** a male in 2020 from West Lake. Map modified with permission of the Alabama Cave Survey.

floor and a lower level ~9 m below the upper level with a mud-cover floor and some breakdown slabs. The western area of the lower level is known as Johnson Hall. Historically, a Grey Bat summer maternity colony roosted in the northwest section of Jones Hall in an area called Bat Lake. East Hall (also called East Room) is the smallest of the three main chambers measuring ~244 m × 61 m. Miller Hall (also called West Room) is the western most chamber and is accessed from Jones Hall through Cooper's Crawl. This chamber measures ~135 m × 300 m with ceilings rarely exceeding 4.6 m and the floor characterized by large blocks of breakdown covered by thick mud. The 6.1-m pit entrances open into a high passage that connects Jones Hall with East Hall. During periods of low water levels in late summer and fall, isolated pools can be found in Jones

Hall and East Hall that gradually diminish in extent until no water is present (Cooper 1975). Miller Hall contains the only permanent water in the cave year-round – West Lake which typically maintains 0.9–1.2 m of water and up to 0.6 m of mud and silt substrate during the dry season. Grey Bats also were known to roost in this section of the cave. During winter and spring, the local water table rises, and the lower levels of the cave completely flood with groundwater becoming inaccessible. Water levels may fluctuate 10 m or more between the wet and dry seasons. Dye tracing has demonstrated that water flows from West Lake in Miller Hall to Jones Hall likely to East Hall and ultimately to Brahan Spring 3.5 km to the southeast of the cave.

Aquatic surveys

We conducted visual encounter surveys of accessible aquatic habitats (isolated pools and phreatic lakes) on 20 occasions between October 2018 and July 2021. Surveys were conducted by 1–5 researchers for 0.75–6.5 person-hours depending on water levels. During low water levels, surveys were conducted on foot in the lower levels of Jones Hall, Miller Hall, and East Hall. During high water levels, surveys were typically limited to the upper levels of Jones Hall and East Hall. We searched for aquatic life with headlamps and handheld dive lights. During high water, we also conducted snorkel surveys in Jones Hall. We made a concerted effort to capture with handheld dipnets and examine all cave crayfish observed. Select crayfish were photographed before measuring total length, carapace length, chelae length and width, and examined reproductive condition. Species identification was based on examination of chelae, gonopods, and body size (as noted above in the Introduction) and aided by photographs of type material in the North Carolina State Museum of Natural Sciences by Guenter Schuster. We also removed and retained a walking leg as a tissue sample preserved in 100% ethanol for genetic analyses. Captured crayfish were released at their point of capture after processing.

Population trends

To investigate whether cave crayfish abundance (i.e., direct visual counts) has changed over time at Shelta Cave, we compiled count data from literature sources spanning 1968–2012, including Cooper (1975), Lee (1987), Hobbs and Bagley (1989), Rheams et al. (1992), McGregor et al. (1994, 1997), Cooper and Cooper (1997), and Miller (2013). These data were combined with visual counts from our recent surveys. We employed generalized linear models (GLMs) with the census counts as the response variable and survey date as the explanatory variable for two time periods: 1) 1968–1975 during Cooper's (1975) regular crayfish surveys for his dissertation work, and 2) 1985–2021. We examined two datasets: a dataset representing visual counts for *O. sheltae* and the second which includes visual counts of all cave crayfishes observed irrespective of species. Because count data often exhibit a Poisson or negative binomial distribution and also can be zero-inflated (Linden and Mantyniemi 2011), we explored the best fit of several different distributions, including zero-inflated and non-zero-in-

flated Poisson, negative binomial, and negative binomial with NB2 parameterization [variance = $\mu(1 + \mu /k)$], using the *glmmTMB* (Brooks et al. 2017) package in R. We developed zero-inflated models using a single zero-inflation parameter but also developed hurdle models that first modeled the binary likelihood that a 0 value is observed and modeled the non-zero observations using a truncated Poisson or negative binomial model. We determined the best fitting models using AICc using the *bblme* package in R (Bolker and R Core Development Team 2017). The best fitting model was used to estimate the overall trend during each time period. We also tested for differences in abundance of each dataset between the two time periods using a Mann-Whitney test after testing for deviations from normality with a Shapiro-Wilk normality test.

DNA extraction, PCR, and sequencing

We extracted DNA from walking legs using the Qiagen DNEasy Blood and Tissue Kit according to the manufacturer's protocol except for a few modifications. Walking legs were manually crushed using a small pestle after addition of Buffer ATL. This was followed by the addition of 40 µL of proteinase K. The sample was incubated overnight at 56 °C, with occasional vortexing while incubating to ensure adequate mixing. After the addition of Buffer AL, the sample was incubated at 70 °C for 10 minutes. Finally, the DNA was eluted using 125 µL of Buffer AE which had been preheated to 70 °C.

Polymerase chain reaction (PCR) was used to amplify two mitochondrial loci, 454 bp of 16S rRNA (*16S*) and 642 bp of cytochrome oxidase subunit I (*CO1*). Each 25 µL PCR reaction consisted of 12.5 µL of GoTaq Colorless MasterMix (Promega), 1.0 µL each of 10 µM forward and reverse primers (Table 1), 7.5 µL of molecular grade water, and 3.0 µL of DNA template. Gel electrophoresis was used to confirm successful PCR amplification using an Axygen gel documentation system. PCR products were cleaned using ExoSAP-IT (Affymetrix) and sequenced in both directions using BigDye chemistry at Eurofins MWG Operon (Louisville, Kentucky) using PCR primers.

Table 1. PCR primers for amplification of two mitochondrial (*16S* and *CO1*) loci in the current study.

Primer	Gene	Sequence (5'-3')	Reference
16Sar	*16S*	CGCCTGTTTATCAAAAACAT	Palumbi (1996)
16Sbr	*16S*	CCGGTCTGAACTCAGATCACGT	Palumbi (1996)
LCO1490	*CO1*	GGTCAACAAATCATAAAGATATTG	Folmer et al. (1994)
HCO2198	*CO1*	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)

Phylogenetic analyses

Forward and reverse sequences were trimmed at the ends based on quality and assembled into contigs in ChromasPro v2.1.8 (Technelysium Pty Ltd, South Brisbane, Australia). Contigs were aligned using MUSCLE (Edgar 2004) in MEGA X version 10.0.5 (Kumar et al. 2018). We checked for the presence of premature stop codons

and indels in *CO1* sequences, which would indicate pseudogene sequences, by converting to amino acid sequences in MEGA X. Novel sequences generated in this study were accessioned into GenBank (accession nos. ON380874–ON380893 for *CO1* and ON383885–ON383904 for *16S*). We also included in our *16S* and *CO1* datasets sequences for other cave and surface cambarid crayfishes available on GenBank (Suppl. material 1: Table S1) to infer phylogenetic placement of *O. sheltae* within Cambaridae and with respect to other cave crayfishes, in particular. This approach has been used in other decapods (e.g., Varela and Bracken-Grissom 2021; Varela et al. 2021). The crayfishes *Astacus astacus* (Linnaeus, 1758) and *Pacifastacus leniusculus* (Dana, 1852) both in the family Astacidae were included as outgroup taxa. We constructed gene genealogies using maximum likelihood (ML) analysis using RAxML-HPC v.9.2.10 (Stamatakis 2014). Optimal models of nucleotide substitution for each locus, including first, second, and third codon positions for *CO1* and *16S*, were determined in PartitionFinder2 (Lanfear et al. 2017) using corrected Akaike's Information Criterion (AICc). ML analyses were conducted under the GTRGAMMA model and rapid bootstrapping algorithm with 10,000 bootstraps. Partitions determined by PartitionFinder2 were included in the analyses. Trees were visualized in FigTree v1.4 (Rambaut 2014). We calculated mean uncorrected pairwise distances between *O. sheltae* and other cave crayfishes occurring in northern Alabama in MEGA X (Kumar et al. 2018).

Results

Aquatic surveys

We observed 20 cave crayfish (mean ± 1 SD: 1.3 ± 1.6 crayfish) during 12 of 20 surveys between October 2018 and July 2021. Eighteen crayfish were identified as *O. australis*. However, two individuals were identified as *O. sheltae* (Fig. 1). During a snorkel survey of North Lake in Jones Hall on 31 May 2019, MLN captured a small crayfish in ~3.7 m of water. The female crayfish measured 36 mm total length and 18 mm carapace length (Fig. 1B). The crayfish lacked first pleopods, possessed narrow and elongate chelae, and lacked prominent spines on the mesial margin of the carpus. All characters, in addition to smaller adult size, are consistent with *O. sheltae*. Developing ova also were observed internally. A second individual was captured in West Lake in Miller Hall on 28 August 2020 by MLN and NS. This form I male measured 30.5 mm total length and 14.4 mm carapace length, possessed narrow, elongate chela, and lacked prominent spines on the mesial margin of the carpus (Fig. 1C). In addition, gonopod shape was consistent with the original description of *O. sheltae* (fig. 1B, C, E, and F in Cooper and Cooper 1997).

Population trends

We assembled cave crayfish count data for 122 surveys spanning from November 1968 through July 2021 including the current study (Suppl. material 2: Table S2). Total cave

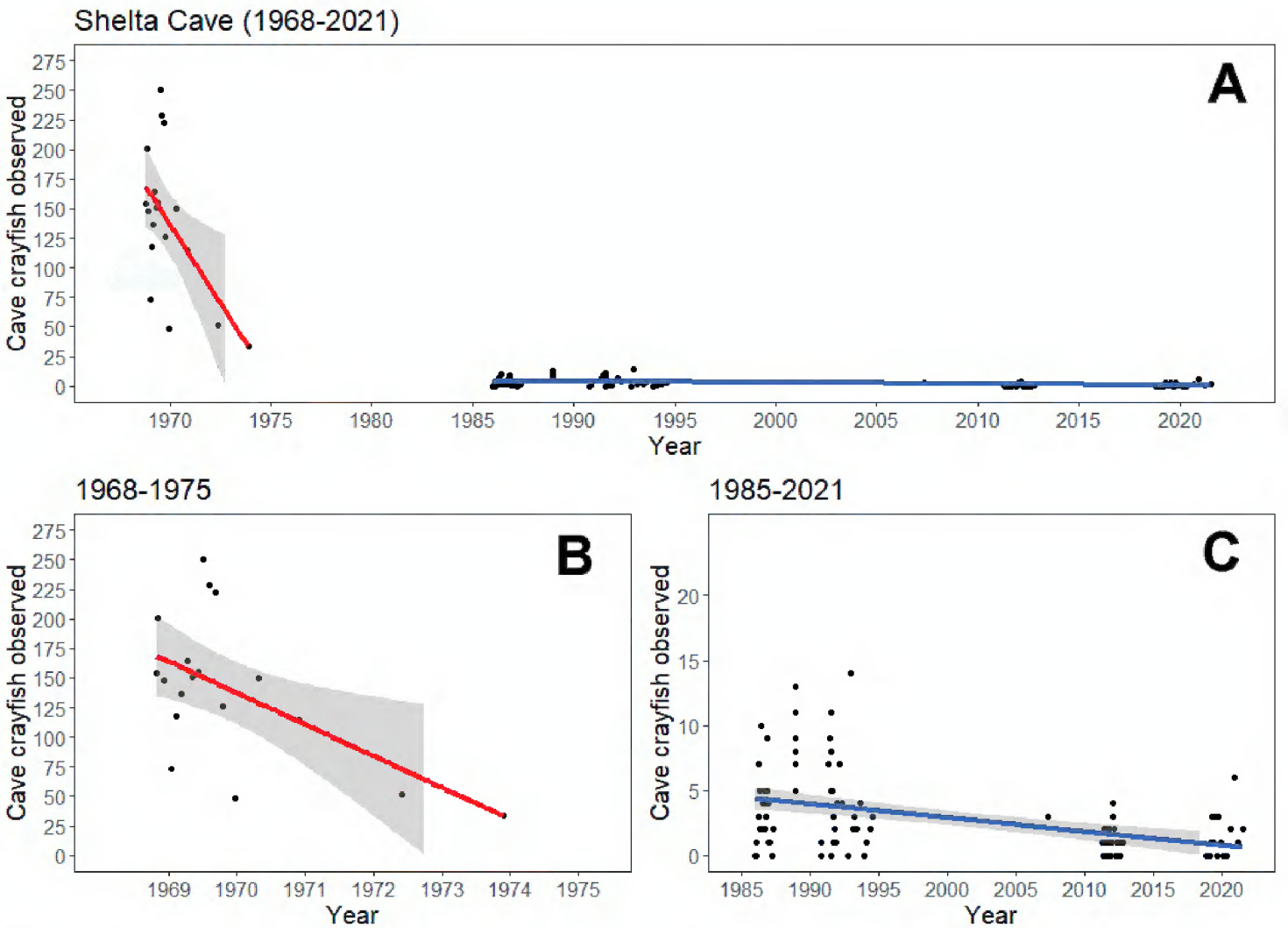

Figure 2. A Trends in visual census counts of all cave crayfishes (*Orconectes australis*, *Cambarus jonesi*, and *C. sheltae*) at Shelta Cave from 1968–2021, with **B** trends during the study of Cooper (1975) and **C** since 1985 also visualized. Note that the scale on the x- and y-axes differ for **B** and **C**.

crayfish abundance averaged 140.4 ± 61.9 crayfish (maximum: 250; minimum: 34) during Cooper's (1975) study over 18 surveys spanning November 1968–November 1973. *Orconectes sheltae* abundance averaged 6.2 ± 6.2 individuals (maximum: 18; minimum: 0) over the same period. Both total cave crayfish (U = 1818, $P < 0.001$; Fig. 2) and *O. sheltae* abundance (U = 1907, $P < 0.001$; Fig. 3) were drastically lower over 101 surveys spanning December 1985–July 2021 compared to the November 1968–July 1975 period. Total cave crayfish abundance averaged 2.8 ± 3.2 crayfish (maximum: 14; minimum: 0) and *O. sheltae* abundance averaged 0.03 ± 0.17 crayfish (maximum: 1; minimum: 0) over this latter period. Best fitting models (negative binomial and negative binomial with ND2 parameterization; Table 2) showed significant declines in abundance between 1968 and 1975 for all cave crayfishes (Fig. 2B) and *O. sheltae* (Fig. 3B).

Phylogenetic analyses

The *16S* and *CO1* ML analyses placed *O. sheltae* in a clade with other troglobiotic *Cambarus* endemic to the Interior Low Plateau karst region in northern Alabama with strong support (Figs 4, 5). This clade includes *C. hamulatus*, *C. jonesi*, *C. laconensis*, *C. pecki*, *C. speleocoopi*, and *O. sheltae*. Mean uncorrected pairwise distances between

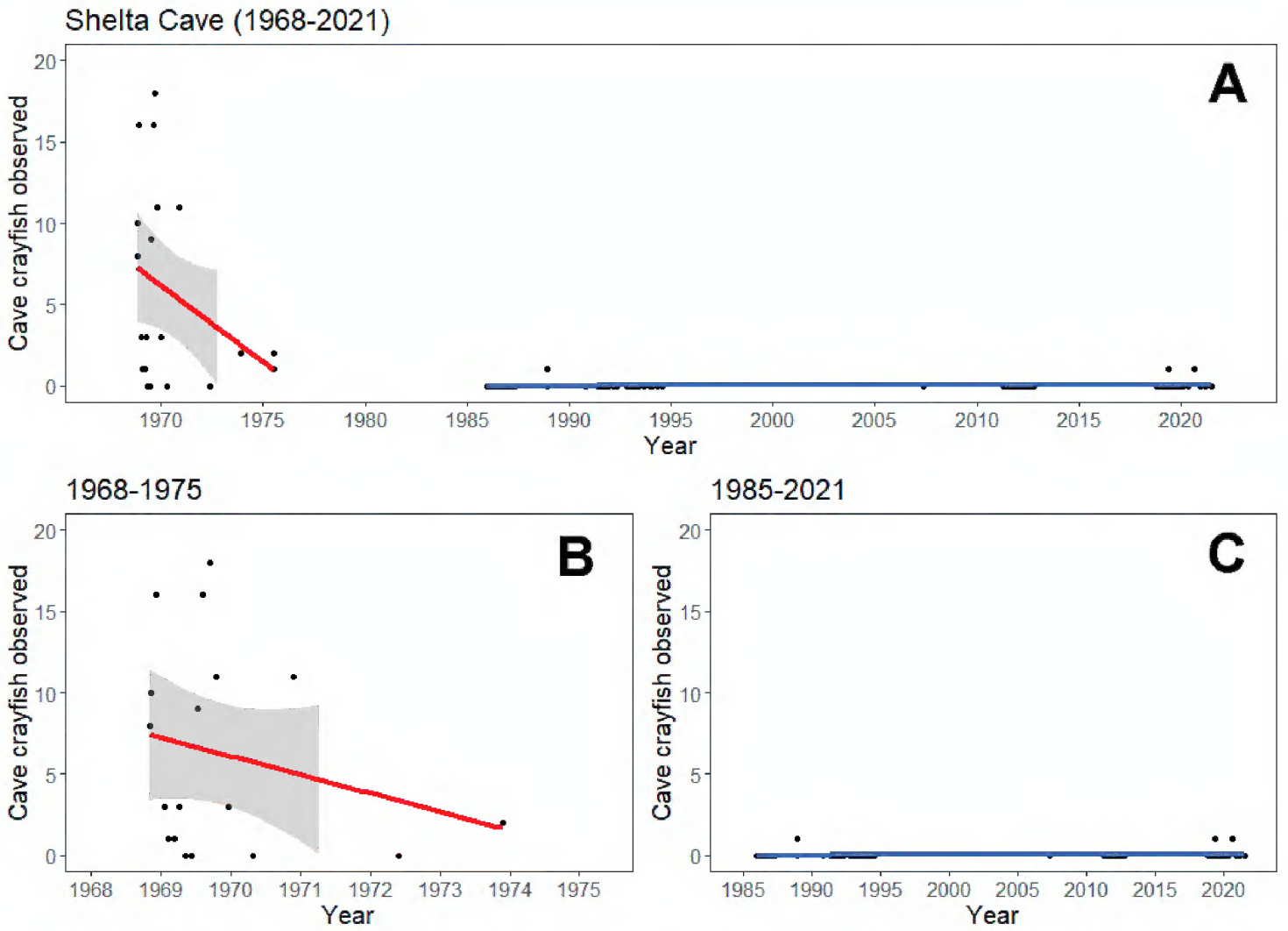

Figure 3. A Trends in visual census counts of *Cambarus sheltae* at Shelta Cave from 1968–2021, with **B** trends during the study of Cooper (1975) and **C** since 1985 also visualized. Note that the scale on the x-axis differs for **B** and **C**.

Table 2. Summary of parameter estimates and AICc for best model distributions (i.e., ΔAICc < 2) comparing abundance (visual census counts) over time (days) at Shelta Cave for the 1968–1975 and 1985–2021 periods for all cave crayfish and for just *Cambarus sheltae*. Zero-inflation (zi.m) model parameters are included. Significance: *** - p < 0.001; ** p < 0.01; * p < 0.05.

Dataset	Model	AICc	Parameters
All cave crayfish			
1968–1975	Negative binomial with NB2	197.1	Intercept: 5.47***; days: -8.39e-04***; k: 7.9; df: 3
	Negative binomial	198.3	Intercept: 5.37***; days: -6.72e-04***; k: 18.2; df: 3
1985–2021	Negative binomial with NB2 parameterization	418.3	Intercept: 2.36***; days: -1.25e-04***; k: 1.7; df: 3
	Zero-inflated hurdle negative binomial with NB2	420.1	Intercept: 2.43***; days: -1.26e-04***; k: 2.1; zi.m. intercept: -3.04; df: 4
Shelta Cave Crayfish			
1968–1975	Negative binomial with NB2	120.1	Intercept: 2.30***; days: -7.45e-04; k: 1.0; df: 3
1985–2021	Gaussian	-66.6	Intercept: -0.018; days: 3.91e-06; df: 3

O. sheltae and other species in this clade ranged 5.1–7.8% and 3.0–6.2% for *CO1* and *16S*, respectively. The other cave-obligate *Orconectes* form a monophyletic group more closely related to other members of *Cambarus* (Figs 4, 5). Mean uncorrected pairwise distances between *O. sheltae* and cave *Orconectes* ranged 10.6–11.9% and 6.8–7.8% for *CO1* and *16S*, respectively.

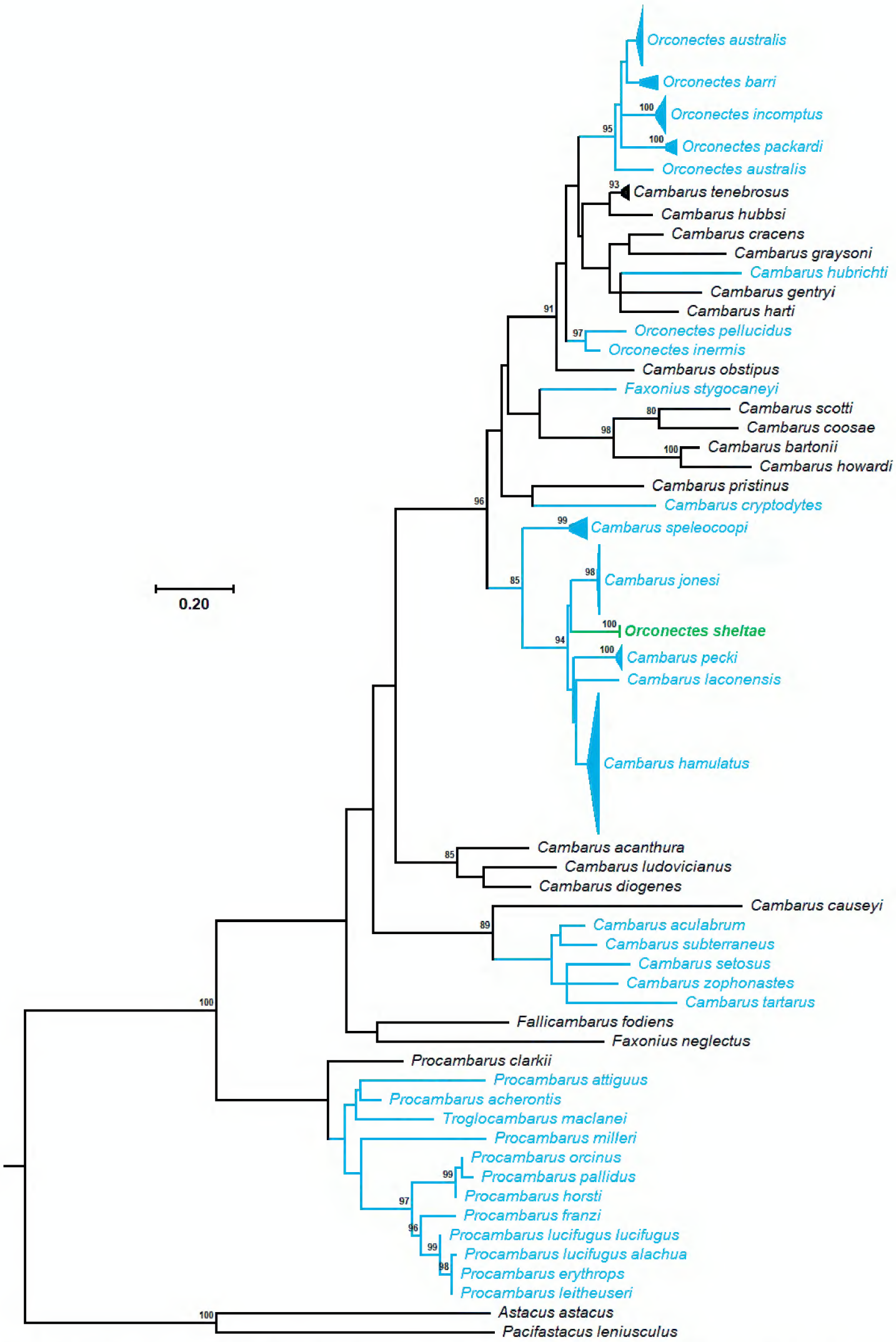

Figure 4. Maximum-likelihood phylogram showing relationships among *Orconectes sheltae* (in green) and other cave (in blue) and surface (in black) cambarid crayfishes inferred from the mitochondrial *CO1* locus. Bootstrap support >80% is shown to the left of the corresponding node.

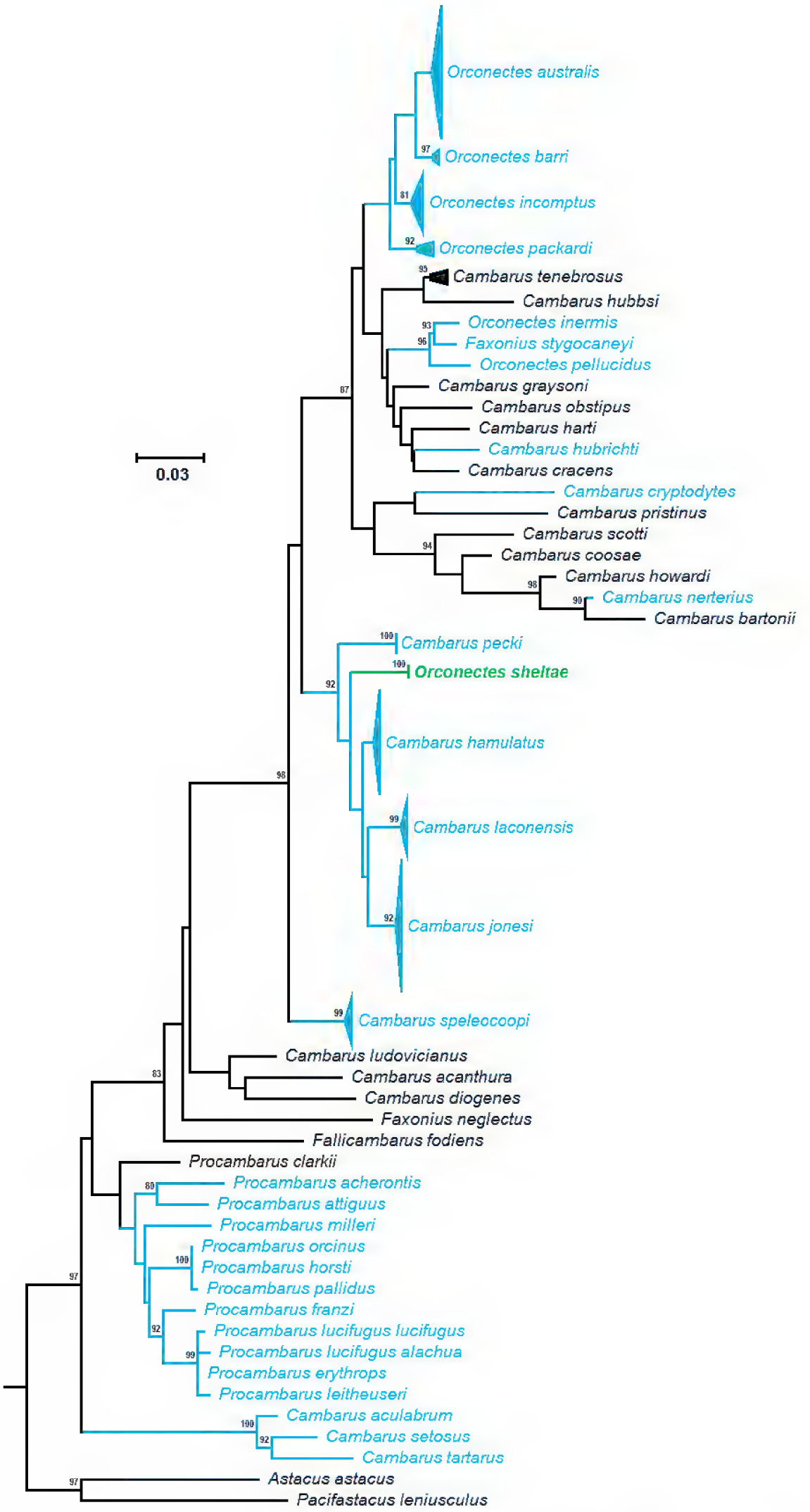

Figure 5. Maximum-likelihood phylogram showing relationships among *Orconectes sheltae* (in green) and other cave (in blue) and surface (in black) cambarid crayfishes inferred from the mitochondrial *16S* ribosomal RNA locus. Bootstrap support >80% is shown to the left of the corresponding node.

Discussion

Rediscovery of *Orconectes sheltae*

The discovery of two individuals of *O. sheltae* during recent surveys in 2019–2020 demonstrates that the species is not yet extinct, as has been hypothesized by past authors (Buhay and Crandall 2005; Elliott 2005; Niemiller and Taylor 2019). However, the species remains critically imperiled and on the brink of extinction, as the population appears to be extremely small in size given only three individuals have been documented since the 1970s and overall cave crayfish abundance since 1985 at Shelta Cave is just ~2% of counts during Cooper's (1975) dissertation work in the late 1960s into the early 1970s. The rediscovery of *O. sheltae* also offers hope that other imperiled stygobiotic species thought to be extirpated may be rediscovered at Shelta Cave in the future. The federally endangered *Palaemonias alabamae* was last observed on 24 November 1973 at Shelta Cave (Cooper 1975; Cooper and Cooper 2011) but has been documented at two new sites in the last 20 years (USFWS 2016; Niemiller et al. 2019). The salamander *Gyinophilus palleucus* was never abundant at Shelta Cave or other caves in and around the metropolitan Huntsville area (Cooper 1968, 1975; Cooper and Cooper 1968, 2011; Niemiller and Niemiller 2020). This top aquatic predator has not been observed since 1968 at Shelta Cave (Cooper 1975). *Orconectes sheltae* co-occurs with two other stygobiotic crayfishes at Shelta Cave. While recent surveys have confirmed the continued persistence of *O. australis*, *Cambarus jonesi* has not been documented since 1988 (Hobbs and Bagley 1989).

Population declines in *O. sheltae* and other stygobionts at Shelta Cave have been linked to impaired water quality and reduction in energy input into the aquatic ecosystem (Hobbs and Bagley 1989; Moser and Rheams 1992; Wilson and Robison 1993; McGregor et al. 1997; Elliott 2000, 2012). Elevated levels of cadmium and the pesticides heptachlor and dieldrin have been detected in the groundwater of Shelta Cave, the latter likely associated with increased urbanization (Hobbs and Bagley 1989; Moser and Rheams 1992; McGregor et al. 1997). The pesticides likely entered via rapid infiltration through epikarst from residential areas located within the recharge basin of the cave system. The aquatic ecosystem likely also was negatively impacted by the loss of a *Myotis grisescens* maternity colony (Elliott 2000, 2012), which was an important source of organic input through deposition of guano and dead individuals. After purchasing the cave in 1967, the National Speleological Society constructed a bat-unfriendly gate in 1968. The colony was estimated at 54,000 bats in 1969, and bats were occasionally observed during the early 1970s (Cooper 1975; Cooper and Cooper 2011); however, the colony had completely disappeared by the late 1970s (Hobbs and Bagley 1989). The gate was replaced with a more appropriate design in 1981 (Hobbs and Bagley 1989) and completely removed and replaced with a high fence around the entrance in the early 2000s (Elliott 2012), but the bat colony has yet to return, and the aquatic ecosystem has yet to recover at Shelta Cave.

Phylogenetic placement and taxonomic implications

The taxonomy of crayfishes in the family Cambaridae has been based historically on morphology; however, phylogenetic relationships and evolutionary histories may be obfuscated by convergent evolution (Crandall and Fitzpatrick 1996; Taylor and Knouft 2006; Breinholt et al. 2012), which may be of particular concern when elucidating phylogenetic relationships in cave-obligate crayfishes (Sinclair et al. 2004; Buhay and Crandall 2009) and cave organisms in general (Christiansen 1961; Culver et al. 1995; Wiens et al. 2003). The taxonomy of Cambaridae continues to be in a state of flux. Several studies have uncovered lack of support for monophyly of several genera (Taylor and Knouft 2006; Johnson et al. 2011; Breinholt et al. 2012; Crandall and De Grave 2017; Stern et al. 2017; Glon et al. 2018), including *Orconectes* (Crandall and Fitzpatrick 1996; Fetzner 1996; Sinclair et al. 2004; Buhay and Crandall 2005, 2008; Owen et al. 2015; Stern et al. 2017). Recently, Crandall and De Grave (2017) presented a phylogeny based on a subset of molecular data generated in Stern et al. (2017) which showed that cave *Orconectes* form a distinct clade more closely related to members of *Cambarus*, while surface *Orconectes* are more closely related to *Barbicambarus*, *Creaserinus*, and other members of *Cambarus*. Consequently, Crandall and De Grave (2017) restricted *Orconectes* to the cave taxa, as the type species of the genus is *Orconectes inermis*, and resurrected the genus *Faxonius* Ortmann, 1905 for the surface-dwelling group. Phylogenetic relationships of cave-obligate cambarid crayfishes based on *16S* and *CO1* datasets estimated in this study are similar to relationships estimated based on a maximum-likelihood analysis of partial mitochondrial (*12S*, *16S*, and *CO1*) and nuclear (*28S*) sequence data by Carroll et al. (2021) and a six-locus dataset (*12S*, *16S*, *CO1*, *18S*, *28S*, and histone *H3*) by Stern et al. (2017).

Our phylogenetic analyses revealed that the genus *Orconectes* as currently recognized is in need of additional taxonomic refinement, as we did not find support for inclusion of *O. sheltae* within *Orconectes*. Cooper and Cooper (1997) placed the Shelta Cave Crayfish in the genus *Orconectes* based primarily on gonopod morphology, and this classification has been followed since its description (e.g., Crandall and de Grave 2017). However, Cooper and Cooper (1997) noted that *O. sheltae* is "quite different from the other troglobitic members of the genus." Specifically, *O. sheltae* i) lacks the first pleopods in females; ii) possesses a broad median trough of the annulus; ii) possesses elongate, narrow chelae, with a long palm and subvertical orientation; iv) has longer terminal elements of the form I male gonopod, with a greater degree of curvature and cephalocaudal flattening of the central projection; v) possesses a great depth of the cephalocaudal axis of the shaft of the gonopod proximal to the base of the central projection; vi) lacks prominent spines on the mesial margin of the carpus; and vii) is small in size, with a maximum carapace length of 19.7 mm. Our molecular results demonstrate that *O. sheltae* is a member of a clade that contains other geographically proximate cave-obligate species in northern Alabama in the genus *Cambarus*. Therefore, we advocate for recognition of this species as *Cambarus sheltae* to more accurately reflect evolutionary relationships in this taxon.

Discordance between gonopodal morphology and genetics in cave crayfishes of northern Alabama is not without precedence. Buhay and Crandall (2009) discovered

that *Procambarus pecki* was in fact also a member of *Cambarus* based on molecular analyses and recognized the species as *Cambarus pecki*. Like *O. sheltae*, *P. pecki* was noted previously to be "disjunct with other members of the genus" and once belonged to its own monotypic subgenus *Remoticambarus* (Hobbs 1972). It is becoming increasingly clear that relying solely on morphological characters is inadequate to infer species' boundaries and taxonomic relationships in cave-obligate crayfishes because of convergent evolution on troglomorphic characters, such as reduction in eye structures, loss of pigmentation, and attenuation of antennae and limb, as well as confounding gonopodal structures. Rather, genetic data paired with geographic information, other ecological data, and diagnosable characters (outlined in Buhay and Crandall 2009) appear to be sufficient for species identification in these stygobiotic crayfishes. It is important to note that past authors have cautioned against relying solely on mitochondrial loci for inferring phylogenetic relationships, as this has the potential to yield inaccurate hypotheses in arthropods (Fontaine et al. 2007; Song et al. 2008; Leite 2012), including crayfishes (Song et al. 2008; Buhay 2009; Schubart 2009), due to factors such as paternal leakage into the mitogenome (e.g., Fontaine et al. 2007; Mastrantonio et al. 2019) and the presence of mitochondrial pseudogenes (Song et al. 2008; Buhay 2009). We followed recommendations by Buhay (2009) and found no evidence of pseudogenes (i.e., premature stop codons and presence of indels) in our *O. sheltae CO1* sequences. We did detect the presence of putative pseudogenes in some *O. australis* sequences, however, and these sequences were excluded from analyses.

Conclusions and recommendations

In this study, we reported on the first observations of *O. sheltae* at Shelta Cave since 1988. The rediscovery of this single-cave endemic crayfish offers optimism that other cave and groundwater species that have not been observed in several decades may still persist but remain at high risk of extinction. In the case of *O. sheltae* and the stygobiotic life at Shelta Cave in general, visual counts during recent surveys remain just a fraction of abundance observed over half a century ago. We generated the first genetic data and conducted the first phylogenetic analysis of *O. sheltae* finding strong support for placement of this species in the genus *Cambarus* with several other cave-obligate *Cambarus* species that occur in northern Alabama.

We offer several recommendations for management, conservation, and future research of *O. sheltae*. First, we propose the establishment of a long-term monitoring program for *O. sheltae* and other stygobiotic life at Shelta Cave to assess trends over time. Our study employed visual encounter surveys that can only be conducted when water levels are low. The use of baited funnel traps may be advantageous to increase detection for stygobiotic crayfishes (Crandall 2016; Fenolio et al. 2017; DiStefano et al. 2020), particularly during periods of high water. The use of environmental DNA (eDNA) for detecting and monitoring groundwater organisms has become increasingly popular in recent years. This approach, which leverages DNA shed by organisms

into their surrounding environment, has been employed with success in caves, springs, and wells for a diversity of groundwater organisms, including salamanders (Goricki et al. 2016, 2017; Voros et al. 2017; Lyons 2019), cavefishes (Lyons 2019; Mouser 2019; White et al. 2020; Mouser et al. 2021), amphipods (Niemiller et al. 2018), and crayfishes (Boyd 2019; Mouser 2019; Boyd et al. 2020; DiStefano et al. 2020; Dooley 2021; Mouser et al. 2021). With the first genetic data and tissues now available for *O. sheltae*, we recommend that a species-specific eDNA assay be developed and an eDNA survey study employed to potentially locate additional sites for this imperiled stygobiotic crayfish. Finally, new water quality analyses are needed to ascertain if contaminants, particularly pesticides and heavy metals, continue to percolate into the groundwater and determine if new threats exist that may impact the survival of the stygobiotic community at Shelta Cave.

Acknowledgements

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Supplementary material 1

Table S1

Authors: Katherine E. Dooley, K. Denise Kendall Niemiller, Nathaniel Sturm, Matthew L. Niemiller

Data type: genetic sequences (docx. file)

Explanation note: **Table S1.** Genbank sequences included in the 16S and CO1 phylogenetic analyses.

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Link: https://doi.org/10.3897/subtbiol.43.79993.suppl1

Supplementary material 2

Table S2

Authors: Katherine E. Dooley, K. Denise Kendall Niemiller, Nathaniel Sturm, Matthew L. Niemiller

Data type: occurrence data (docx. file)

Explanation note: **Table S2.** Count data of cave crayfishes at Shelta Cave, Madison Co., Alabama from 1968 to 2021.

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